Epitope mapping of the immunodominant proteins of bullous skin disorders

PhD THESIS

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1. Introduction

Autoimmune blistering disorders affect the skin and mucous membranes. The blistering reduces the body's defense against infection and dehydration, thus endangering the patient's life as well. *Pemphigus* is a group of organ-specific autoimmune bullous skin diseases. The group characterized by the skin and / or mucosal intraepidermal blistering. The two main types of *pemphigus* are *pemphigus vulgaris* (PV) and *pemphigus foliaceus* (PF). In case of PV, mucosus and mucocutaneous versions are known, in the former only mucosal symptoms can be observed, while in the latter the skin and mucous membranes are also affected.

The immunopathogenesis of the disease is not clarified yet, although more and more information is available regarding its mechanism. In PV pathogenic autoantibodies are expressed against desmoglein 1 (Dsg1) and desmogelin 3 (Dsg3) desmosomal transmembrane proteins which are the main target antigens [1]. In addition to the production of autoantibodies the autoantigen specific T-cells also play an important role in the development and progression of the disease [2], and the activation of the $CD4^+$ T-cells depends on specific human leukocyte antigen (HLA) class-II alleles expressed on antigen presenting cells [3].

Identification and detailed analysis of the immunodominant B- and T-cell epitope regions of the protein antigens have an impact in understanding the immunopathology and development of the disease and in the design of novel diagnostics. Use of synthetic peptides as epitopes may be advantageous for diagnostic purposes, because peptides are smaller molecules than protein antigens; their preparation in many cases is faster, simpler and more cost effective. Furthermore, synthetic peptides often have better solubility and stability characteristics when compared to proteins.

In PV, identified linear B-cell epitopes or epitope regions have been described within the extracellular part of the protein Dsg3 [4-8], while in the protein Dsg1 only conformational B-cell epitopes have been described [9]. After thorough review of the literature of this topic we have concluded that for the precise identification of linear B-cell epitope regions systematic epitope mapping has to be carried out on both proteins.

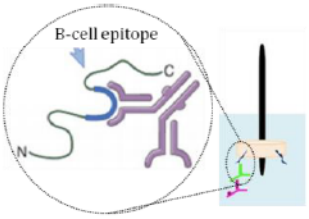
Several research groups dealt with the identification of T-cell epitopes of the protein Dsg3 as well [10-14]. For the identification synthetic peptides were used which do not require and probably do not go through uptake and processing, and can be presented directly by HLA class I and II molecules *via* peptide loading and exchange catalyzed by a specific HLA class II molecule [15]. In the experiments more Dsg3 T-cell epitope region were identified, and

various research groups have received different results, which is understandable given that PV is difficult to systematically study because of the relatively small number of accessible untreated donors, different stage of the disease, or because of the different phenotype, cytokine profile and epitope specificity of the cells used in the experiments [10, 11].

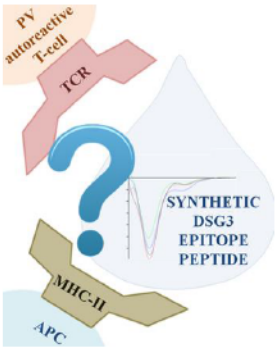
It can be assumed that the *in vitro* T-cell response ability of the synthetic epitope peptides can be affected by the solubility, stability and solution conformation of peptides [16]. However, these properties have not been studied in case of PV T-cell epitopes.

2. Aims

The main aims of my studies were:



1. Systematic epitope mapping of linear B-cell epitopes of the PV immunodominant proteins (Dsg1 and Dsg3) with the synthesis and immunoserological analysis of overlapping pin-bound peptides.



2. Determination of structure - activity relationships on synthetic Dsg3 T-cell epitope region peptides. Design, synthesis, secondary structure analysis and *in vitro* T-cell activity studies of the peptides.

3. Methods

Chou-Fasman secondary structure and Eisenberg hydrophobicity predictions: The number of Dsg1 and Dsg3 protein related peptides being synthesized on non-cleavable pins were rationalized based on Chou-Fasman secondary structure prediction and Eisenberg hydrophobicity prediction studies.

Peptide synthesis on non-cleavable pins: For the B-cell epitope mapping of the extracellular part of Dsg1 and Dsg3 proteins pentadecapeptides overlapping in five amino acid residues were synthesized with Fmoc/*t*Bu strategy on non-cleavable pins.

Solid phase peptide synthesis: Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives were synthesized on Rink Amide MBHA resin with solid

phase peptide synthesis using Fmoc/*t*Bu strategy. Synthetic peptides were characterized by analytical RP-HPLC, ESI-MS and amino acid analysis.

Enzyme-Linked Immunosorbent Assay (ELISA): To detect the interaction between the serum autoantibodies and the pin-attached overlapping peptides modified ELISA was performed.

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA): PBMCs (peripheral blood mononuclear cells) were isolated from the whole blood samples of PV patients and healthy donors, and then stimulated with the synthetic peptides. The *in vitro* T-cell response inducing activity of Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives was studied by sandwich ELISA measurement of the IFN-γ concentration of the stimulated PBMC's supernatants.

Determination of in vitro cytotoxicity with MTT assay: *In vitro* cytotoxic effect of the Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives was determined using MTT assay.

Electronic circular dichroism spectroscopy (ECD): Solution conformation of the Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives was studied by ECD spectroscopy.

4. Results and discussion

4.1. Mapping of linear B-cell epitope regions of the Dsg1 and Dsg3 proteins using pin-bound synthetic peptides

B-cell epitope mapping using a series of overlapping synthetic peptides selected on the basis of the secondary structure prediction of the immunodominant protein can be a very efficient way to identify linear antigenic determinants. In our work, pin-bound overlapping pentadecapeptides based on the Dsg1 and Dsg3 protein sequence were synthesized using Fmoc/*t*Bu strategy, then the recognition of the synthetic peptides by autoantibodies of PV, PF patient and healthy donor serum samples were tested with ELISA optimized by us.

4.1.1. Modelling of the Dsg1 and Dsg3 protein sequence with synthetic overlapping peptides

Following the Chou-Fasman secondary structure prediction and Eisenberg hydrophobicity prediction 45 and 47 peptides - based on the Dsg1 and Dsg3 protein sequence - overlapping in 5 amino acid residues were synthesized in duplicates on hydroxypropyl methacrylate pins. The synthesized peptides covered 83% of the extracellular domains of the proteins [s1].

4.1.2. Amino acid analysis of the pin-bound peptides

Analysis of the pin-bound peptides can only be solved by invasive methods, after which the analyzed peptides can no longer be used in the tests. Therefore, we could not solve the characterization of all the peptides. Therefore, to demonstrate the success of the synthesis and to determine the peptide content of the pins, during and after the synthesis representative pins were selected for amino acid analysis. Based on the results, each analyzed pin contained average of 40 nmole peptide, corresponding to the expected composition, which is an appropriate amount for antigen-antibody interaction [s1].

4.1.3. Determination of the epitope regions on the basis of peptides serum autoantibody recognition

The results of the immunoserological analysis of pin-bound overlapping peptides were compared with the results published on the subject. According to our results, we have identified five new linear B-cell epitope regions within the extracellular part of the Dsg1 protein (Dsg1/86-110, Dsg1/196-220, Dsg1/226-250, Dsg1/326-340, Dsg1/486-520) and four epitope regions within the extracellular part of the Dsg3 protein, among which two were new (Dsg3/375-399, Dsg3/446-460) and two were shorter sequence (Dsg3/64-78, Dsg3/330-344) than the previously described ones [s1]. The location of the epitope regions within Dsg1 and Dsg3 proteins illustrated by 3D homology models are shown in Figure 1.

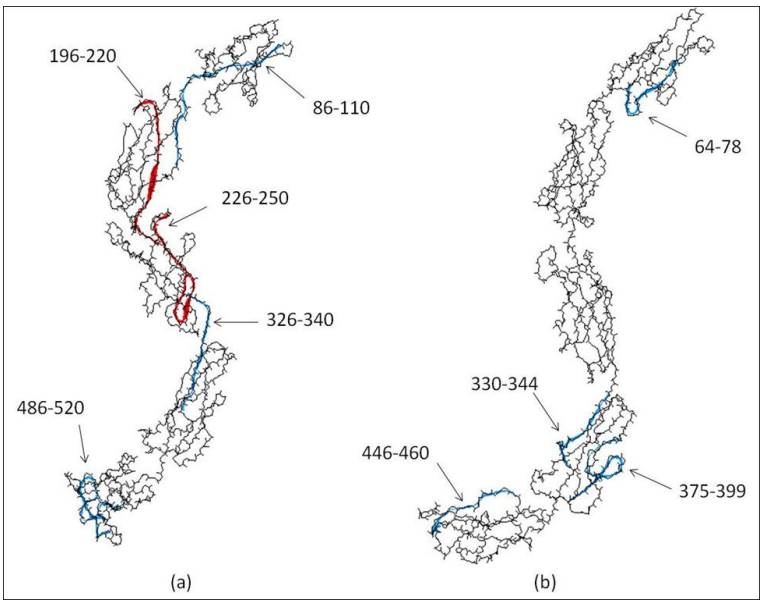


Figure 1. Localization of experimentally determined B-cell epitope regions on 3D homology model of human Dsg1 (a) and Dsg3 (b) proteins. Red ribbons: 5/5 Dsg1 positive and 3/3 Dsg1,3 positive patients serum autoantibody recognition of the regions. Blue ribbons: in case of Dsg1 peptides 4/5 Dsg1 positive and 3/3 Dsg1,3 positive patients serum autoantibody recognition, while in case of Dsg3 peptides 5/6 Dsg3 and Dsg1,3 positive patients serum autoantibody recognition of the regions [s1].

Our results showed that serum autoantibodies of patients, previously identified as Dsg1 or Dsg3 positive, are able to recognize sequences of both Dsg1 and Dsg3 proteins [s1]. These results may be related to the fact that more proteins will be affected in the progress of these autoimmune blistering skin disorders, which can be explained with the high homology of these proteins.

4.1.4. The homology of the identified epitope regions with other proteins

Protein structures homologous to the identified epitope regions have been searched in specific databases. We have found that the Dsg1 and Dsg3 B-cell epitope regions homologous mainly to the other desmoglein proteins, apart from some hypothetical and bacterial protein hits with lower scores [s1]. The Dsg1/196-220 epitope region showed homology with a human transaldolase 1 epitope peptide [s1]. This fact needs to be considered in future studies.

4.2. Structure – activity relationship studies of the Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives

4.2.1. The Dsg3 T-cell epitope region peptides and their N-terminally truncated derivatives

Four 17mer T-cell epitope region (based on literature data) peptide (Dsg3/189-205, Dsg3/206-222, Dsg3/342-358, Dsg3/761-777) and their 11 new, *N*-terminally truncated derivatives (Dsg3/190-205, Dsg3/192-205, Dsg3/194-205, Dsg3/208-222, Dsg3/210-222, Dsg3/343-358, Dsg3/345-358, Dsg3/347-358, Dsg3/763-777, Dsg3/764-777, Dsg3/766-777) have been synthesized by solid phase peptide synthesis using Fmoc/*t*Bu strategy [s2].

4.2.2. Chemical, structural and in vitro functional characterization of the synthetic peptides

The products were chemically characterized by analytical RP-HPLC, ESI-MS and amino acid analysis. Results have shown that every peptide uniform and corresponds to the expected composition [s2]. The lyophilised peptide content was 60-70%.

The stability, *in vitro* cytotoxicity, solution conformation and *in vitro* T-cell response inducing activity of the peptides have been studied.

4.2.2.1. Stability, solubility and in vitro cytotoxicity of the synthetic peptides

Each peptide was stable for 48 hours at 37°C in the cell culture medium used in the *in vitro* tests [s2]. Most of the peptides were soluble in RPMI-1640 cell culture medium. Based on the results of the MTT assay, none of the peptides were cytotoxic in the concentration used for the *in vitro* stimulation of PBMCs [s2].

4.2.2.2. Solution conformation of the Dsg3 T-cell epitope region peptides and their N-terminally truncated derivatives

Solution conformation of the synthetic peptides was measured by ECD spectroscopy in solvent trifluoroethanol (TFE), TFE/H_2O (1:1, v/v) and H_2O. Based on our results, from the 17mer peptides the formation of the ordered structures of Dsg3/761-777 peptide depends more on the type of the solvent used, compared to the Dsg3/189-205, Dsg3/206-222 and Dsg3/342-358 peptides [s2]. The *N*-terminal truncation of the 17mer Dsg3 peptides caused gradually smaller ordered structure content in their conformer population in TFE. The differences in the conformer populations of the peptide series best can be seen in the spectra recorded in TFE/H_2O (1:1, v/v) mixture. In the conformer populations of the Dsg3/192-205 peptide and the Dsg3/761-777 peptide series the unordered structure elements have higher ratio (in addition to isolated turn structures), which indicate low solvent resistance, which might suggest high backbone flexibility [s2].

4.2.2.3. The in vitro T-cell response inducing activity of the synthetic peptides

The *in vitro* T-cell response inducing activity of the Dsg3 T-cell epitope region peptides and their *N*-terminally truncated derivatives was studied by the determination of IFN-γ cytokine concentration of the supernatants of PBMC cultures isolated from the whole blood of 3 PV patients and 3 healthy donors. *In vitro* T-cell response was identified over 50±25 pg/ml IFN-γ concentration range, which was determined from the IFN-γ concentrations of supernatants from untreated PBMCs [s2]. Empty symbols in Figure 2 represent ELISA results with more than 25% standard deviation. Based on our experience, high SD values can occur when using supernatants for IFN-γ determination [s2]. Nevertheless, based on our results, using synthetic Dsg3 peptides *in vitro* T-cell response differences between PV patients and healthy donors can be observed. Using Dsg3/192-205, Dsg3/342-358, Dsg3/763-777 and Dsg3/764-777 peptides as synthetic antigens we could distinguish the PV patients from healthy donors (Figure 2, highlighted in blue) [s2]. Instead of individual peptides, peptide sets can also be applied. Based on our results it is worth considering the further study of the following peptides as a synthetic antigen set: Dsg3/192-205, Dsg3/342-358, Dsg3/763-777 and Dsg3/764-777 [s2].

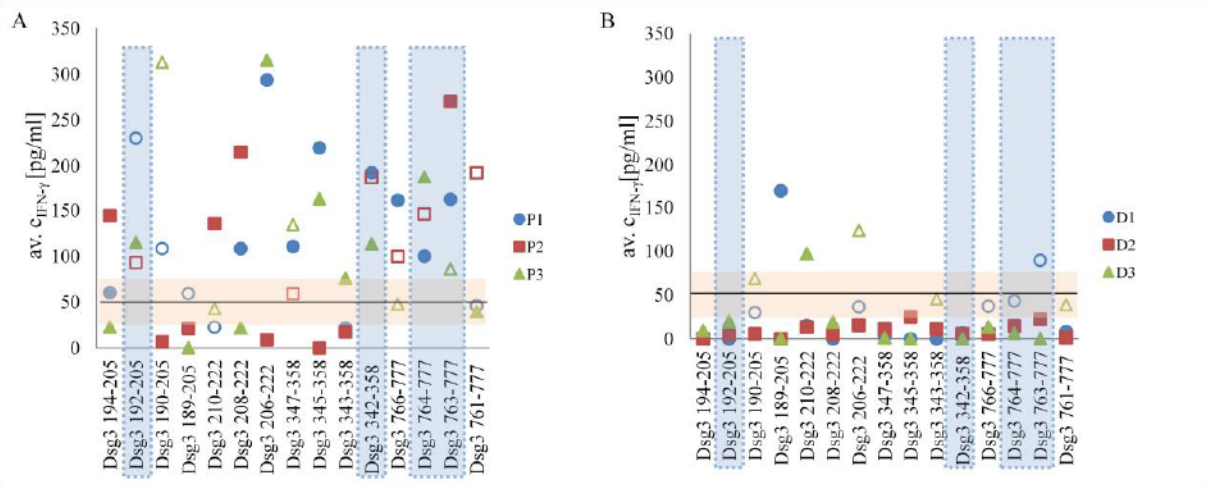


Figure 2. Average IFN-γ concentration of 2-2 parallel measurements from supernatants of three PV patients (A) and three healthy donors (B) determined by ELISA after 20 hours of *in vitro* PBMC stimulation with 25 μM synthetic peptide [s2]. (av.: average, filled symbols: SD<25%, unfilled symbols: SD>25%, yellow horizontal area: 50±25 pg/ml IFN-γ concentration range, blue highlighting: clear distinction can be made between PV patients and healthy donors)

4.2.3. Structure - activity relationships of T-cell epitope region peptides

Comparing the *in vitro* activity with data from secondary structure analysis we would suggest that the solvent sensibility and high backbone flexibility of the synthetic peptides can be a considerable issue for differential *in vitro* PBMC stimulation for PV patients and healthy donors [s2].

5. Summary

1. During my PhD work we have successfully identified five new linear B-cell epitope regions within the extracellular part of the Dsg1 protein.

2. In the extracellular part of the Dsg3 protein two new and two shorter (than described in the literature) linear B-cell epitope regions have been identified.

3. We have determined the solution conformation of 4 Dsg3 T-cell epitope region peptides and their 11 *N*-terminally truncated derivatives using ECD spectroscopy. The results were in good agreement with the results of Chou-Fasman secondary structure prediction.

4. We have determined that using the Dsg3/192-205, Dsg3/342-358, Dsg3/763-777 and Dsg3/764-777 peptides to stimulate PBMC cultures, we could clearly distinguish the PV patients from healthy donors. These peptides may be suitable for use as synthetic antigens in the T-cell based diagnosis of PV.

5. Based on the structure - activity relationships of the Dsg3 T-cell epitope region peptides we have observed remarkable differences between the *in vitro* T-cell stimulating ability of Dsg3/192-205, Dsg3/763-777 and Dsg3/764-777 peptides on PV patients and

healthy donors. This phenomenon can be related to the presumed backbone flexibility of these synthetic peptides. Based on our results, we would suggest that the solvent sensibility and high backbone flexibility of the peptides can be a considerable issue for differential *in vitro* PBMC stimulation ability.

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[s1] H. Szabados, Sz. Bősze, P. Silló, S. Kárpáti, F. Hudecz, K. Uray: „The mapping of linear B-cell epitope regions in the extracellular parts of the desmoglein 1 and 3 proteins: Recognition of immobilized peptides by *pemphigus* patients' serum autoantibodies." *J. Pept. Sci.* **2013**. 19: 84-94.

[s2] H. **Szabados**, K. Uray, Z. Majer, P. Silló, S. Kárpáti, F. Hudecz, S. Bősze: „Characterization of Desmoglein-3 Epitope Region Peptides as Synthetic Antigens: Analysis of their *in vitro* T-cell Stimulating Efficacy, Cytotoxicity, Stability and their Conformational Features," *J. Pept. Sci.* **2015**. (accepted for publication)

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H. Szabados, K. Uray, P. Silló, F. Hudecz, S. Kárpáti, Sz. Bősze: „Analysis of *in vitro* T-cell responses of PBMCs from patients with *pemphigus vulgaris* and healthy controls using desmoglein 3 peptides and peptide conjugates as antigens." *Immunology*, **2012**. 137, S1, 467.

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